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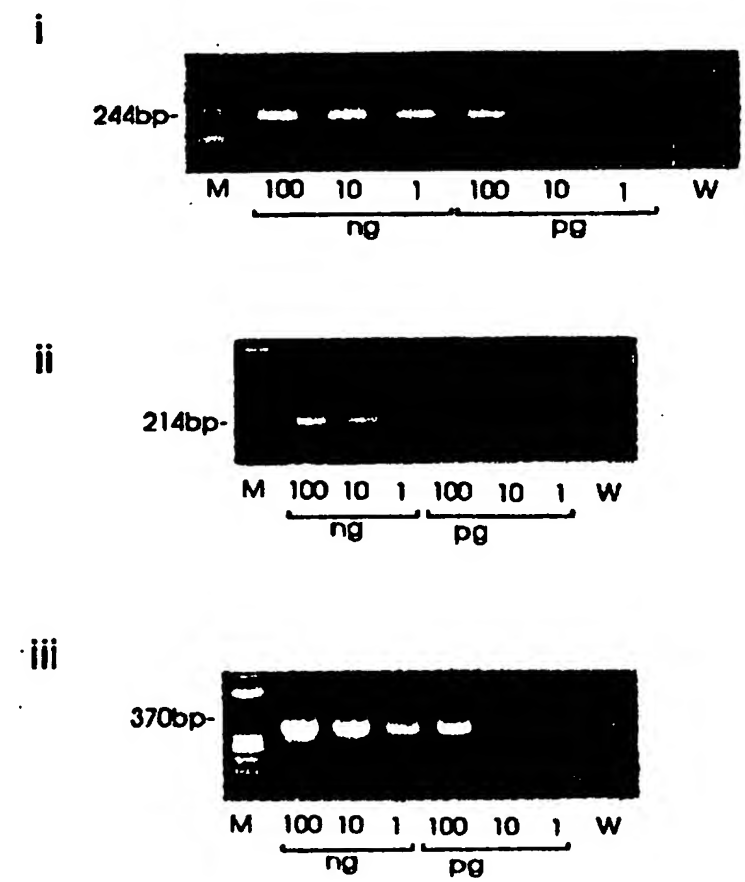
(57) Abstract

A method of determining whether a human patient has a tumour or whether a tumour has metastasised comprising the steps of (1) obtaining a sample of tissue from the patient, the said tissue being one that does not normally contain a cytokeratin 20 (CK20) gene product and (2) determining whether a cytokeratin 20 (CK20) gene product is present in said tissue sample. Once it has been determined whether a human patient has a tumour or is likely to develop metastatic disease from such a tumour, the physician can decide on an appropriate course of treatment.



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DETECTING TUMOURS

The present invention relates to methods of detecting tumours including metastatic disease in a patient, particularly metastatic disease of epithelial cell tumour origin, more particularly disseminating colon carcinoma.

The development and growth of malignant tumours or cancers commonly results in the release of some of the cancerous cells from the developing tumour into the blood or other body fluids. These cells are then transported to other parts of the body where they may become implanted and set up secondary tumours or metastases, thus leading to a general dissemination or spread of the original cancer that is responsible for production of the primary tumour. The process of metastasis may commence at quite an early stage in the development and growth of the primary tumour, and in fact it is metastases, frequently haematogenous metastases produced by cancer tumour cells circulating in the peripheral blood, that determine the outcome of the disease for most patients.

It is important to detect such cancer cells in body fluids, particularly peripheral blood, as this may aid in diagnosing the original cancer and monitoring the disease. In particular, detection of such cancer cells in the peripheral blood is an indicator of the likelihood of metastatic disease and is useful to the physician in deciding upon a suitable course of treatment.

The number of such cancer or tumour cells circulating in such body fluids, particularly peripheral blood, is generally very small and they cannot therefore be distinguished and readily detected by routine microscopy. Techniques for their detection therefore need to be highly sensitive but must remain specific.

Because of the huge number of blood cells compared with potential cancer or tumour cells it is very important that any marker that is used to detect the said cancer or tumour cells is not found in the normal blood cells.

Various methods have been described previously in an attempt to develop methods of detecting metastatic disease by analysing blood and bone marrow samples for cancer cells. Moss & Sanders (1990) *J. Clin. Oncol.* 8, 736-740 describes the detection of neuroblastoma cells in blood using monoclonal antibodies reactive against unidentified neuroblastoma cell antigens.

Sawyers *et al* (1990) *Proc. Natl. Acad. Sci. USA* 87, 563-567 discloses a method of detecting chronic myelogenous leukaemia (CML) cells in the blood of a patient using the polymerase chain reaction (PCR). In this case cellular mRNA was extracted from blood samples and cDNA made using reverse transcriptase (RT). Thus, a RT-PCR was used to amplify cDNA corresponding to mRNA transcribed from the abnormal gene found in CML.

GB 2 260 811 A discloses a general method for the diagnosis or monitoring of cancer of a malignant tumour using a RT-PCR. In this case, melanoma cells were detected in blood of a patient using a RT-PCR. Specific oligonucleotide primers were used to amplify tyrosinase mRNA. Tyrosinase is not expressed in normal blood but is expressed at a relatively high level in some melanoma cells (which are derived from melanocytes); however, analysis of tyrosinase gene expression is complicated by the presence of tyrosinase-related protein genes and the method is limited because some melanomas lack tyrosinase expression (amelanotic tumours).

Burchill *et al* (1994) *Int. J. Cancer* 57, 671-675 describes neuroblastoma cell detection by RT-PCR for tyrosine hydroxylase mRNA.

It will be appreciated that melanoma, neuroblastoma and CML are tumours arising from highly specialised cell types and, in the case of CML, arising from a cell containing chromosomal abnormality. Some of the most prevalent cancers arise from less specialised epithelial cells, for example breast cancer and colon carcinoma. Clearly, any method of detecting cells in the blood derived from such epithelial cell tumours must rely on the marker to be detected *not* being expressed in normal blood.

Cytokeratins (CKs) are components of mammalian cell cytoskeleton and constitute a multigene family of proteins (see Nagel (1988) *Am. J. Surg. Pathol.* 12, (suppl. 1), 4-16) and Moll *et al* (1982) *Cell* 31, 11-24 for reviews). CKs are expressed predominantly in epithelial cells where they show strict lineage and differentiation-associated patterns of expression. Malignant cells generally retain the CKs of their progenitor cell type and have been used previously to characterise neoplastic cells of epithelial origin.

Traweek *et al* (1993) *Am. J. Pathol.* 142, 1111-1118 have used RT-PCR to detect the expression of CK8, CK18 and CK19 in various tissues. CK8 and CK18 are expressed in all tissue that has been studied including peripheral blood mononuclear cells, aspirated bone marrow cells and lymph nodes. Some of these cells are components of normal blood and so CK8 and CK18 are expressed in normal blood and, consequently, are of no use in analysing blood samples for the presence of cells derived from an epithelial cell tumour.

Although Traweek *et al* apparently did not detect CK19 gene activity in

normal peripheral blood and bone marrow, they found that the CK19 activity in stromal cells caused problems and that CK19 expression was detected in lymph nodes. Datta *et al* (1994) *J. Clin. Oncol.* 12, 475-482 have used RT-PCR to analyse the expression of CK19 in breast cancer patients and, apparently, no gene expression was detected in the blood of patients at stages I, II or III but only ten of twenty-six patients with metastatic disease gave a positive result using the RT-PCR test. However, a low frequency of CK19 expression was found in normal blood indicating illegitimate transcription of CK19 mRNA or the presence of CK19-expressing cells in normal blood or bone marrow. Furthermore, as discussed in more detail in the Examples of the present invention, we have found CK19 expression using RT-PCR in six out of fifteen control blood samples.

Thus, CK19, like CK8 and CK18, is not a suitable target for detection of tumour cells in peripheral blood.

Surprisingly, we have found that CK20 is not expressed in normal blood samples. CK20 is expressed, for example, in a high proportion of colorectal carcinomas, stomach cancers, mucinous ovarian adenocarcinomas and transitional cell carcinomas. Thus, it is an object of the present invention to provide methods for detecting these and other tumours and, in particular, metastatic disease, by detecting the presence of CK20 gene expression in the blood or bone marrow or other suitable tissue of a patient.

One aspect of the present invention provides a method of determining whether a human patient has a tumour or whether a tumour has metastasised comprising the steps of (1) obtaining a sample of tissue from the patient, the said tissue being one that does not normally contain a

cytokeratin 20 (CK20) gene product and (2) determining whether a cytokeratin 20 (CK20) gene product is present in said tissue sample.

By "determining whether a tumour has metastasised" we include determining whether any tumour cells are found at a site remote from a primary tumour whether or not those tumour cells are found in a further tumour.

Thus, the method includes determining the likelihood that a tumour has spread or is spreading or will spread in a patient.

It will be appreciated that, in essence, the invention comprises determining whether a tissue sample from a patient contains a cytokeratin 20 (CK20) gene product, particularly a tissue sample which does not normally contain a cytokeratin 20 (CK20) gene product.

By "CK20 gene product" we include CK20 mRNA and CK20 protein.

It will be appreciated by a person skilled in the art that the human CK20 gene, as is the case for many human genes, is polymorphic and therefore that many allelic forms occur. Thus, by "CK20 gene" we include all allelic forms. Different allelic forms can be readily detected by comparing one CK20 gene or mRNA sequence with another, for example by DNA sequencing or restriction fragment length polymorphism (RFLP) analysis.

We have found unexpectedly that a CK20 gene product is not present in blood or bone marrow from a human who does not suffer from a tumour or, particularly, in whom a tumour has not metastasised. Thus, it is preferred that the tissue sample is blood or bone marrow. Blood is readily obtained from the patient using venepuncture whereas bone marrow is

obtained using standard aspiration techniques (for example by placing a needle into the iliac bone and aspirating). Purged bone marrow is suitable. Alternatively, lymph nodes, peripheral stem cell harvests, urine and cystectomy samples may be used. Thus, we include urine in the term "tissue sample". A suitable sample also includes polymorphonuclear cells such as neutrophils.

When we state that a CK20 gene product is not present in blood or bone marrow from a human who does not suffer from a tumour we mean that the amount of any CK20 gene product is so low that it cannot be detected, at least by presently available techniques. The amount of CK20 gene product present in the tissue (such as blood) of a human patient who is determined by the method of the invention to have CK20 gene product present in said tissue, compared to a human who does not have a CK20 gene product (as defined), is at least two-fold higher, preferably at least 10-fold higher, more preferably at least 100-fold higher and most preferably at least 1000-fold higher.

It is preferred if the tumour is an epithelial cell tumour.

There are many epithelial cell-derived tumours including breast carcinoma colorectal carcinoma, stomach adenocarcinoma, mucinous ovarian adenocarcinoma, all bladder carcinoma including dysplasia and transitional cell carcinoma. The vast majority of mucinous ovarian and colorectal adenocarcinomas express CK20; greater than 75% of stomach and gall bladder adenocarcinomas contain CK20-expressing cells; and more than half of pancreatic adenocarcinomas contain CK20-expressing cells.

It is less preferred if the tumour is a lung tumour.

A high proportion of Merkel cell carcinomas and transitional cell carcinomas express CK20.

Thus, the method of the invention is particularly suited to determine whether a patient has, or is likely to develop metastatic disease from, mucinous ovarian and colorectal adenocarcinomas.

Determination of colorectal adenocarcinoma and metastasis therefrom are particularly preferred.

It is preferred that the patient has not suffered a local trauma or has not undergone surgery both of which may result in the release of epithelial cells into the blood.

Presence of a cytokeratin 20 gene product can be determined in a tissue sample using various techniques. Conveniently, it is determined by detecting CK20 messenger RNA (mRNA). Although, in principle, CK20 mRNA can be detected in a tissue sample by hybridising a specific nucleic acid probe to the mRNA (such as an antisense RNA or antisense oligonucleotide, the said probe being labelled with a readily-detectable moiety for example, a fluorescent dye or a radionuclide), the sensitivity of such a method may not allow the detection of the very small amount of CK20 mRNA that is present in a tissue sample. For example, a 5 ml blood sample may contain only tens of CK20-expressing cells derived from the epithelial cell tumour but tens of thousands of cells in total. Thus, it is preferred that the mRNA is detected by, first, making a complementary DNA (cDNA) copy of the CK20 mRNA and, second, amplifying the cDNA using DNA amplification methods.

In a particularly preferred embodiment the following steps are used:

1. A tissue sample is obtained from the patient. Conveniently, 2 ml of blood is removed from the patient and frozen at -80°C.

2. Total cellular RNA is extracted from the blood sample, for example using standard techniques described in Sambrook *et al* (1989) *Molecular Biology, a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA. Alternatively, commercially available kits can be used such as Ultraspec™ RNA (a trade mark) available from Biogenesis, Bournemouth, UK.

3. The mRNA is converted into cDNA using suitable oligonucleotide primers, deoxynucleotides and an enzyme with reverse transcriptase activity. The oligonucleotide for making cDNA can be, for example, either:

   a) An oligo-dT primer which hybridises to the polyA tail of mRNA;

   b) Random short sequences, such as random hexamers, which prime cDNA synthesis of the total RNA; or

   c) Oligonucleotides specific for CK20 which prime cDNA synthesis from CK20 mRNA.

4. Optionally any residual chromosomal DNA is removed using a nuclease such as DNase I.

5. Oligonucleotide primers are selected which direct specific amplification of the CK20 cDNA. A PCR is performed using these oligonucleotides, a thermal-stable DNA polymerase and deoxynucleotides.

6. Optionally, further oligonucleotides are selected which direct specific amplification of a DNA fragment chosen from the inter-primer region defined in step 5 and a further PCR (a "nested" PCR) is carried out.

7. The product of the PCR reaction of step 5 or 6 is detected using agarose gel electrophoresis and ethidium bromide staining of the DNA.

Nucleotide sequences of CK20 cDNAs are shown in Figures 5 to 7. The cDNA and gene sequence described in Moll *et al* (1993) *Differentiation* **53**, 75-93 is incorporated herein by reference as are the sequences and information from the relevant database submissions described in the legends to Figures 5 to 7. The approximate position of the introns (in the gene) are marked. It is preferred if oligonucleotides suitable for DNA amplification comprise a sequence selected from the sequence shown in any one of Figures 5 to 7 such that the first oligonucleotide is capable of hybridizing to one exon and the second oligonucleotide is capable of hybridizing to another exon. Preferably the oligonucleotide primers are between 10 and 50 nucleotides in length, more preferably between 14 and 30 nucleotide long, most preferably around 20 nucleotides long.

It is preferred if the oligonucleotide primers can hybridize to all alleles of the CK20 gene. Regions of the CK20 gene and cDNAs common to all alleles can be determined by comparing the sequences of CK20 genes and cDNAs such as those shown in Figures 5 to 7.

Preferred oligonucleotide primers comprise or consist of the sequence 5'-CAGACACACGGTGAACTATGG-3' (SEQ ID No 1) and 5'-GATCAGCTTCCACTGTTAGACG-3' (SEQ ID No 2). Further preferred

oligonucleotide primer pairs are (all listed 5′ → 3′):

CTCCTGGAATCTCCAATGG (SEQ ID No 3) and
GCATTTTGCAGTTGAGCATCC (SEQ ID No 4);

CTCCAATGGATTTCAGTCG (SEQ ID No 5) and
AATTTGCAGGACACACCGAGC (SEQ ID No 6);

CTAAATGACCGTCTAGCGAGC (SEQ ID No 7) and
TCCACATTGACAGTGTTGCCC (SEQ ID No 8);

CCAACTCCAAACTTGAAGTGC (SEQ ID No 9) and
TCCACATTGACAGTGTTGCCC (SEQ ID No 10); and

TGGGCAACACTGTCAATGTGG (SEQ ID No 11) and
TCCATGTTACTCCGAATCTGC (SEQ ID No 12).

It is well known that the sequence at the 5′ end of the oligonucleotide need not match the target sequence to be amplified.

It is usual that the PCR primers do not contain any complementary structures with each other longer than 2 bases, especially at their 3′ ends, as this feature may promote the formation of an artifactual product called "primer dimer". When the 3′ ends of the two primers hybridize, they form a "primed template" complex, and primer extension results in a short duplex product called "primer dimer".

Internal secondary structure should be avoided in primers. For symmetric PCR, a 40-60% G+C content is often recommended for both primers, with no long stretches of any one base. The classical melting temperature

calculations used in conjunction with DNA probe hybridization studies often predict that a given primer should anneal at a specific temperature or that the 72°C extension temperature will dissociate the primer/template hybrid prematurely. In practice, the hybrids are more effective in the PCR process than generally predicted by simple T_m calculations.

Optimum annealing temperatures may be determined empirically and may be higher than predicted. *Taq* DNA polymerase does have activity in the 37-55°C region, so primer extension will occur during the annealing step and the hybrid will be stabilized. The concentrations of the primers are equal in conventional (symmetric) PCR and, typically, within 0.1- to 1-μM range.

As an alternative to detecting the product of DNA amplification using agarose gel electrophoresis and ethidium bromide staining of the DNA, it is convenient to use a labelled oligonucleotide capable of hybridising to the amplified DNA as a probe. When the amplification is by a PCR the oligonucleotide probe hybridises to the interprimer sequence as defined by the two primers. The oligonucleotide probe is preferably between 10 and 50 nucleotides long, more preferably between 15 and 30 nucleotides long. The probe may be labelled with a radionuclide such as ^{32}P, ^{33}P and ^{35}S using standard techniques, or may be labelled with a fluorescent dye. When the oligonucleotide probe is fluorescently labelled, the amplified DNA product may be detected in solution (see for example Balaguer *et al* (1991) "Quantification of DNA sequences obtained by polymerase chain reaction using a bioluminescence adsorbent" *Anal. Biochem.* **195**, 105-110 and Dilesare *et al* (1993) "A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantitation" *BioTechniques* **15**, 152-157.

Any of the DNA amplification protocols can be used in the method of the invention including the polymerase chain reaction, QB replicase and ligase chain reaction. The polymerase chain reaction is particularly preferred because of its simplicity.

The polymerase chain reaction may, if desired, be carried out *in situ*, or at least in whole tissue samples isolated from the patient, using the methods described in Komminoth *et al* (1994) *Pathol. Res. Pract.* **190(1)**, 1017-1025, incorporated herein by reference; and Komminoth *et al* (1994) *Verhandlungers der Deutschen Gesellschaft fur Pathologic* **78**, 146-152, incorporated herein by reference.

In principle it is possible to detect the presence of a CK20 gene product by determining whether the sample contains any CK20 protein. This is most conveniently achieved using antibodies that react specifically with CK20. For example, monoclonal antibody K_s 20.8 reactive against CK20 can be purchased from Cymbus Bioscience Limited, Southampton, UK or other suitable monoclonal antibodies can be made using methods well known in the art. For example, suitable monoclonal antibodies to CK20 may be prepared using the techniques described in *Monoclonal Antibodies: a manual of techniques*, H. Zola (CRC Press, 1988) and in *Monoclonal Hybridoma Antibodies: Techniques and Applications*, J.G.R. Hurrell (CRC Press, 1982).

Conveniently, the antibody is labelled with a readily detectable marker such as a radionuclide or fluorescent dye. Suitable radionuclides include ^{99m}Tc, ^{123}I and ^{32}P. Suitable fluorescent dyes include fluorescein.

Once it has been determined, according to the methods of the invention whether a human patient has a tumour or is likely to develop metastatic

disease from such a tumour, the physician can decide on an appropriate course of treatment. Other methods may supplement the present method in order to reach a diagnosis.

A further aspect of the invention provides a kit of parts comprising oligonucleotide primers capable of amplifying a cytokeratin 20 (CK20) cDNA, deoxynucleotides and a DNA polymerase.

The kit may further comprise means for extracting RNA suitable primers for making CK20 cDNA, an enzyme with reverse transcriptase activity, and means for detecting an amplified DNA product.

The invention will now be described in more detail with reference to the following Figures and Examples in which:

Figure 1 shows the products of RT-PCR for CK8, 19 and 20 mRNA on 1 pg to 100 ng of total mRNA isolated from RT112 (CK8), MCF7 (CK19) and HT29 (CK20) cell lines. A single band of 244, 214 and 370 bp respectively was identified after separation of products in an agarose gel and staining with ethidium bromide. There was an increase in the intensity of this band with increasing RNA concentration.

M = molecular weight markers, W = water control.

Figure 2 shows the products of RT-PCR for CK8(i), 19(ii) and 20(iii) mRNA separated by agarose gel electrophoresis and stained with ethidium bromide in 6 control bloods (1-6).

C = positive control for CK8, 19 or 20 mRNA detection. W = water negative control. M = molecular weight markers.

Figure 3 shows the products of RT-PCR for CK20 mRNA separated by electrophoresis and stained with ethidium bromide in 6 control bone marrow samples (i). RT-PCR for GAP-DH in the same RNA samples (ii).

C = positive control of RNA extracted from HT29 cells. W = water negative control. M = molecular weight markers.

Figure 4 shows the products of RT-PCR for CK20 mRNA separated by agarose gel electrophoresis and stained with ethidium bromide in blood samples spiked with 1 to 10^5 HT29 cells. A single 370 bp band was identified when as few as 100 cells per ml of whole blood were analysed (3). No band was identified in unspiked blood (O). RT negative samples showed no amplified band (ii).

+C = positive control of RNA extracted from HT29 cells. W = water negative control. M = molecular weight markers.

Figure 5 shows the nucleotide sequence of part of a human CK20 mRNA with the amino acid sequence of the encoded polypeptide composed of the exon sequence taken from Accession X73501, (bases 1 to 18061).

Author: Zimbelmann, R; Title: Direct Submission; Journal: Submitted (14 Sep 1993) to the EMBL/GenBank/DDBJ databases. R. Zimbelmann, German Cancer Research Center, Division of Cell Biology, Im Neuenheimer Feld 280, 69254 Heidelberg, FRG.

Intron positions are shown with a ‖.

Figure 6 shows the nucleotide sequence of part of a human CK20 mRNA

with the amino acid sequence of the encoded polypeptide composed of the exon sequence taken from as in

Authors: Moll, R., Zimbelmann, R., Goldschmidt, M.D., Keith, M., Laufere, J., Kasper, M., Koch, P.J. and Franke, W.W; Title: The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas; Journal: Differentiation 53(2), 75-93 (1993).

Intron positions are shown with a ‖.

Figure 7 shows the nucleotide sequence of part of a human CK20 mRNA taken from Accession X73502

Reference: 1 (bases 1 to 1461); Authors: Calnek, D. and Quaroni, A.; Title: Differential localization by *in situ* hybridization of distinct keratin mRNA species during intestinal epithelial cell development and differentiation; Journal: Differentiation 53(2), 95-104 (1993); Medline: 93366035.

Reference: 2 (bases 1 to 1461); Author: Quaroni, A.; Title: Direct Submission; Journal: Submitted (07 Sep 1993) to the EMBL/GenBank/DDBJ databases. A. Quaroni, Cornell University, 724 A Vet. Res. Tower, Section of Physiology, Cornell University, Ithaca, NY 14853, USA.

Example 1: Detection of CK20-expressing epithelial cells in human blood

Materials and methods

Cell Lines. The three well-characterised human cell lines used in the study were the transitional cell carcinoma-derived RT112 cell line, the breast adenocarcinoma MCF-7 cell line and the colonic adenocarcinoma HT29 cell line. MCF-7 and HT29 cells express CK8, CK18 and CK19 (Moll *et al* (1982) *Cell* **31**, 11-24), whereas RT112 express CK8, CK18, CK19 along with other CK isotypes characteristic of bladder epithelial cells (Wu *et al* (1982) *Cell* 31, 693-703). CK20, the most recently identified CK isotype, is expressed by HT29 cells, but not by MCF-7 cells (Moll *et al* (1992) *Am. J. Pathol.* **140**, 427-447). All cell lines were maintained in a 1:1 mixture of DMEM and RPMI 1640 media supplemented with 5% foetal bovine serum and passaged using 0.25% trypsin in versene (0.02% EDTA).

Blood and bone marrow samples. Normal blood or bone marrow samples were obtained from volunteers aged between 18 to 45 years. Samples were taken into EDTA, aliquoted into 2 ml volumes and frozen at -80°C until required for RNA extraction.

RNA extraction. Total cellular RNA was extracted from cell lines, normal whole blood, normal bone marrow or spiked normal blood using Ultraspec™ RNA (Biogenesis, Bournemouth, UK) according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR). The RT-PCR method used was based on that for the detection of neuroblastoma

cells (Burchill *et al* (1994) *Int. J. Cancer* 57, 671-675). Briefly, dilution curves of RNA were DNase treated and reverse transcribed to produce cDNA using a random hexamer primer. RT products were amplified by PCR for CK8, CK19 or CK20 (primer sequences are given in Table 1). RT-PCR products were analysed by agarose gel electrophoresis and ethidium bromide staining. Reverse transcriptase negative controls (RT-ve) in which reverse transcriptase enzyme was omitted were included for all RT-PCR reactions. Water negative controls (W) contained all components for the RT-PCR reaction but no targeted RNA. Where appropriate, positive controls (+C) of RNA extracted from HT29, MCF7 or RT112 cells were included. Molecular weight markers (ϕX 174 RF DNA/Hae III, Gibco BRL, Paisley, Scotland or 123 bp ladder, Pharmacia, Milton Keynes, UK) were included on all agarose gels.

The quality of RNA was confirmed by amplification of cDNA for GAP-DH or 18S probed Northern blot analysis. All primers were purchased from Oswell DNA Services (Edinburgh, Scotland).

Table 1. Primer sequences used for PCR amplification of CK8, CK19 and CK20

	Sense primer	Antisense primer
CK8	AACAACCTTAGGCGGCAGCT (SEQ ID No 13)	GCCTGAGGAAGTTGATCTCG (SEQ ID No 14)
CK19	GCGGGACAAGATTCTTGGTG (SEQ ID No 15)	CTTCAGGCCTTCGATCTGCAT (SEQ ID No 16)
CK20	CAGACACACGGTGAACTATGG (SEQ ID No 1)	GATCAGCTTCCACTGTTAGACG (SEQ ID No 2)

Primer sequences for PCR were selected using the Dieffenbach Selection Programme. Primers were located within different exons and were either 20, 21 or 22 mers.

Specificity of RT-PCR. RT-PCR products were separated on agarose gels and Southern blotted onto nylon membrane (Hybond N^+, Amersham, UK). Filters were hybridised with a gamma ^{32}P end-labelled oligonucleotide probe, the sequence of which lay between each primer set. The nucleotide sequence of RT-PCR products was confirmed by dideoxy chain termination sequencing (Sequenase, USS, Canada).

Cell spiking. Cell spiking experiments were used to test the potential sensitivity of this technique for detection of colon carcinoma cells in blood. Known numbers of HT29 cells were added to whole blood samples, mRNA extracted and RT-PCR for CK20 performed. To 2 ml aliquots of whole blood 10 to 1 x 10^6 cells were added; an unspiked blood sample was included in each experiment. RNA (100 pg) from HT29 cells was included as a positive control.

Results

RT-PCR detection of bladder, breast and colon carcinoma cells. RT-PCR for CK8 generated a single 244 bp band identified on ethidium bromide stained agarose gels (Fig 1,i). This fragment was confirmed by Southern blot analysis and sequencing (data not shown) to be the fragment of CK8 which lies between the two primers used for PCR. The band was detected in 10 pg-100 ng of total RNA from RT112 cells.

RT-PCR for CK19 generated a single 214 bp band identified on ethidium bromide stained agarose gels (Fig 1,ii) which was confirmed to be CK19 by Southern blot analysis and sequencing (data not shown). This band was detected in 100 pg-1 ng of total RNA from MCF7 cells.

RT-PCR for CK20 generated a single band of 370 bp (Fig 1,iii). This

band was confirmed by Southern hybridisation and sequence analysis (data not shown) and detected in 100 pg of total RNA from HT29 cells.

In all three cases there was an increase in band intensity with increasing amounts of RNA (Fig 1). No transcripts were identified in water control samples (Fig 1) or RT negative samples (results not shown).

Control blood and bone marrow analysis. In 8/9 and 6/15 control blood samples analysed CK8 and CK19 RT-PCR products were identified under the described conditions. Southern blotting confirmed amplified bands were CK8 and CK19 RT-PCR products. In 15/15 control blood samples analysed, CK20 was undetectable by ethidium bromide staining or Southern blot hybridisation. RT-PCR results for CK8(i), CK19(ii) or CK20(iii) are shown in Fig 2 for six control bloods, 6/6 were positive for CK8, 3/6 for CK19 and 0/6 for CK20. RT-PCR for CK20 in 6/6 normal bone marrow samples showed no amplified bands (Fig 3,i). The integrity of bone marrow RNA samples was confirmed by RT-PCR for GAP-DH (Fig 3,ii).

Cell spiking. In HT29 cell spiking experiments it was possible to detect down to 100 HT29 cells diluted in 2 ml of whole human blood (Fig 4,i). The 370 bp band generated was shown by Southern blotting to hybridise to a ^{32}P end-labelled oligonucleotide probe specific for CK20 and confirmed by sequence analysis to be that of CK20 (results not shown). No RT-PCR products were detected in whole blood alone (Fig 4,i 0). RT-PCR products were not identified in reverse transcriptase negative samples (Fig 4,ii).

Since CK8 and CK19 were expressed in a high proportion of normal peripheral blood samples (88% and 40% respectively) neither would be

suitable targets for detection of tumour cells in peripheral blood.

CK20 mRNA was not detected in any normal blood or bone marrow samples examined suggesting it to be the CK of choice for detection of carcinomas of epithelial origin. CK20 has been detected in almost all cases of colorectal adenocarcinomas by immunohistochemistry and is a useful target for the detection of disseminating colon carcinoma by RT-PCR.

Example 2: Determining whether a patient has an epithelial cell tumour

5 ml of blood is removed from the patient. RNA is produced from the blood as described in Example 1 and a RT-PCR is carried out using the CK20-specific primers as described in Example 1. If a CK20-specific DNA product is amplified it suggests that the patient may have an epithelial cell tumour. Further confirmatory tests may be performed in order to reach a diagnosis.

Example 3: Determining whether a patient with colorectal carcinoma tumours is likely to develop metastases

5 ml of blood is removed from the patient. RNA is produced from the blood as described in Example 1 and a RT-PCR is carried out using the CK20-specific primers as described in Example 1. If a CK20-specific DNA product is amplified it suggests that the patient is likely to develop metastatic disease disseminated from the colorectal tumour. Further confirmatory tests may be performed in order to reach a diagnosis.

CLAIMS

1. A method of determining whether a human patient has a tumour or whether a tumour has metastasised comprising the steps of (1) obtaining a sample of tissue from the patient, the said tissue being one that does not normally contain a cytokeratin 20 (CK20) gene product and (2) determining whether a cytokeratin 20 (CK20) gene product is present in said tissue sample.

2. A method according to Claim 1 wherein the tumour is an epithelial cell tumour.

3. A method according to Claims 1 or 2 wherein the tissue is blood or bone marrow.

4. A method according to any one of Claims 1 to 3 wherein the epithelial cell tumour is any one of colorectal adenocarcinoma, stomach adenocarcinoma, mucinous ovarian adenocarcinoma, bladder carcinoma, gall bladder adenocarcinoma and transitional cell carcinoma.

5. A method according to Claim 4 wherein the epithelial cell tumour is colorectal adenocarcinoma.

6. A method according to any one of the preceding claims wherein the presence of the cytokeratin 20 (CK20) gene product is determined by detecting cytokeratin 20 (CK20) messenger RNA (mRNA).

7. A method according to Claim 6 wherein the cytokeratin 20 messenger RNA (mRNA) is copied into complementary DNA (cDNA).

8. A method according to Claim 7 wherein the complementary DNA (cDNA) is amplified.

9. A method according to Claim 8 wherein the complementary DNA (cDNA) is amplified using the polymerase chain reaction (PCR).

10. A method according to any one of Claims 6 to 9 wherein genomic DNA is removed from or cleaved in the said sample prior to detecting cytokeratin 20 (CK20) messenger RNA (mRNA).

11. A method according to Claim 9 or 10 wherein the primers used in the polymerase chain reaction (PCR) comprise or consist of the sequences 5'-CAGACACACGGTGAACTATGG-3' (SEQ ID No 1) and 5'-GATCAGCTTCCACTGTTAGACG-3' (SEQ ID No 2).

12. A kit of parts comprising oligonucleotide primers for amplifying a cytokeratin 20 (CK20) cDNA, deoxynucleotides and a DNA polymerase.

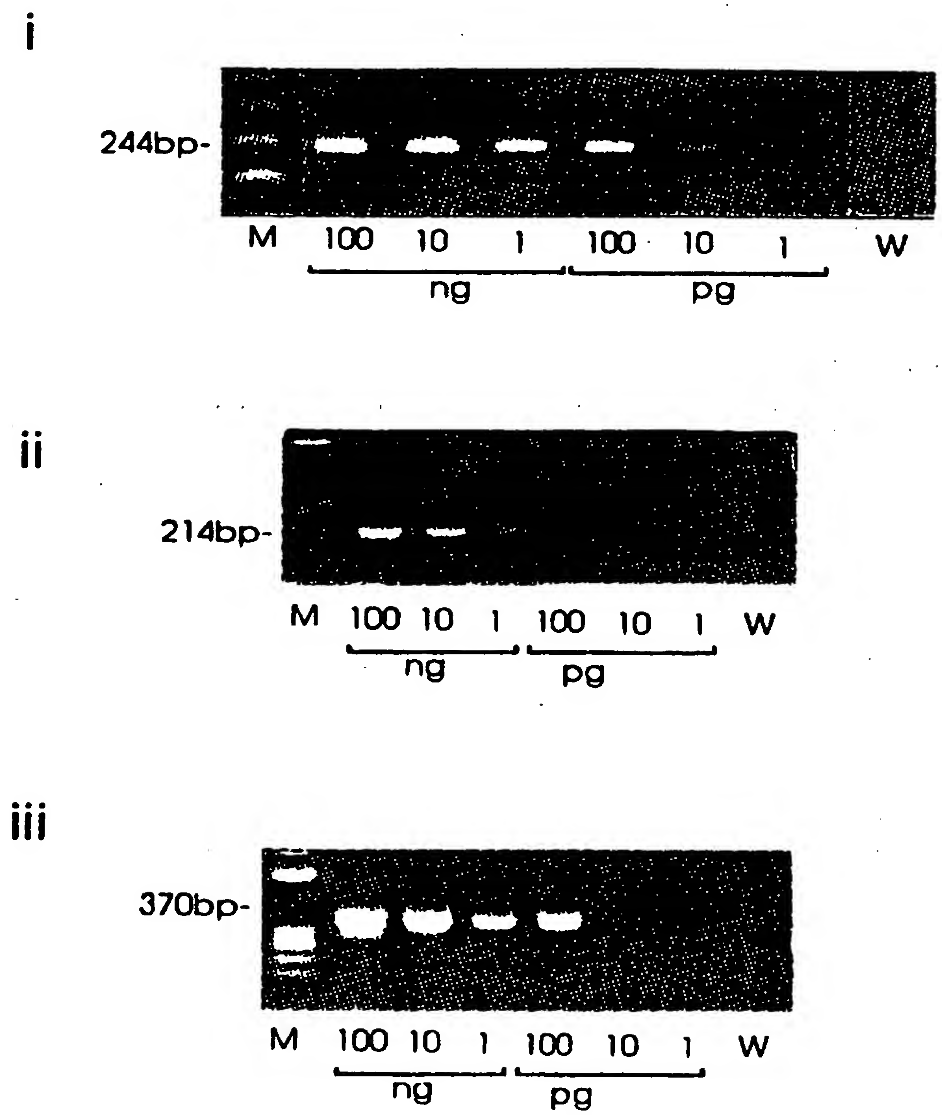
i
244bp-
M 100 10 1 100 10 1 W
ng
pg
ii
214bp-
M 100 10 1 100 10 1 W
ng
pg
iii
370bp-
M 100 10 1 100 10 1 W
ng
pg

Fig.1

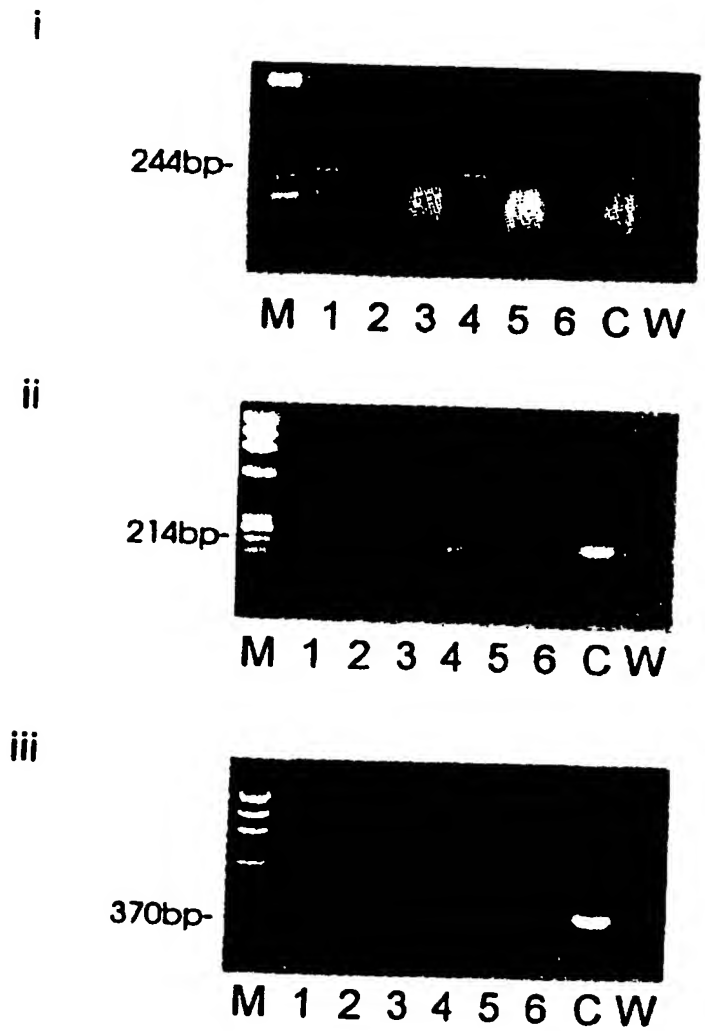

Fig. 2

i

370bp-

M +C W 0 10 10^2 10^3 10^4

ii

370bp-

M +C W 0 10 10^2 10^3 10^4

Fig. 3

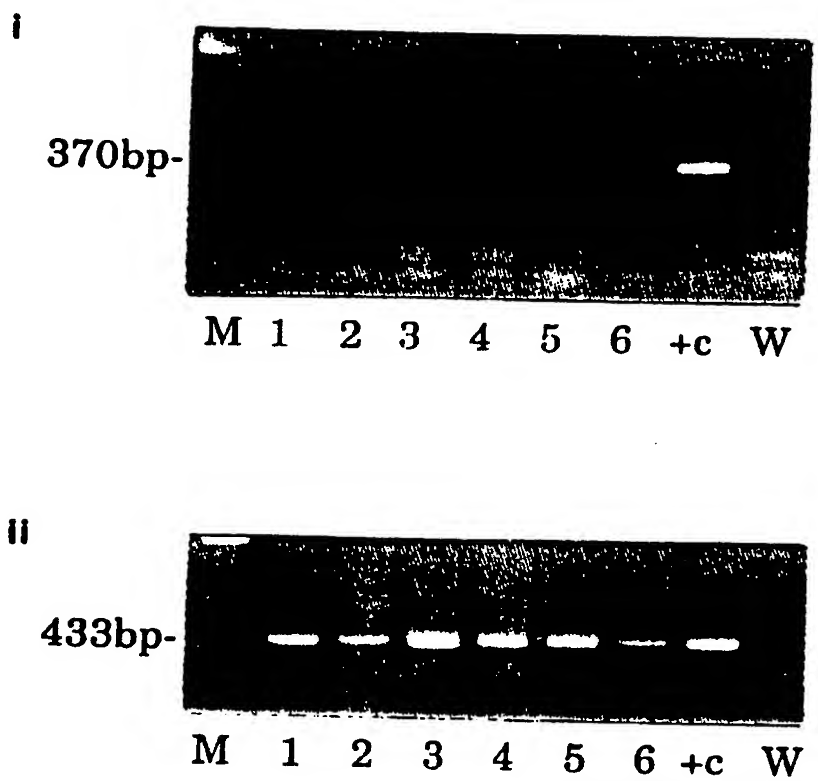

Fig. 4

```
1    T

2    GTCAGCAGAG GAGGAGTTTC TTGCCTGTGG ACTTCATAAA AGGCTAGCTC

52   AACACCCTCC ATGAGACACA CTCTGCCCCA ACCATCCTGA AGCTACAGGT

102  GCTCCCTCCT GGAATCTCCA ATGGATTTCA GTCGCAGAAG CTTCCACAGA
                           M  D  F  S  R  R  S  F  H  R

152  AGCCTGAGCT CCTCCTTGCA GGCCCCTGTA GTCAGTACAG TGGGCATGCA
     S  L  S  S  S  L  Q  A  P  V  V  S  T  V  G  M  Q

202  GCGCCTCGGG ACGACACCCA GCGTTTATGG GGGTGCTGGA GGCCGGGGCA
      R  L  G  T  T  P  S  V  Y  G  G  A  G  G  R  G  I

252  TCCGCATCTC CAACTCCAGA CACACGGTGA ACTATGGGAG CGATCTCACA
      R  I  S  N  S  R  H  T  V  N  Y  G  S  D  L  T

302  GGCGGCGGGG ACCTGTTTGT TGGCAATGAG AAAATGGCCA TGCAGAACCT
     G  G  G  D  L  F  V  G  N  E  K  M  A  M  Q  N  L

352  AAATGACCGT CTAGCGAGCT ACCTAGAAAA GGTGCGGACC CTGGAGCAGT
      N  D  R  L  A  S  Y  L  E  K  V  R  T  L  E  Q  S

402  CCAACTCCAA ACTTGAAGTG CAAATCAAGC AGTGGTACGA AACCAACGCC
      N  S  K  L  E  V  Q  I  K  Q  W  Y  E  T  N  A

452  CCGAGGGCTG GTCGCGACTA CAGTGCATAT TACAGACAAA TTGAAGAGCT
     P  R  A  G  R  D  Y  S  A  Y  Y  R  Q  I  E  E  L

502  GCGAAGTCAG||ATTAAGGATG CTCAACTGCA AAATGCTCGG TGTGTCCTGC
      R  S  Q  I  K  D  A  Q  L  Q  N  A  R  C  V  L  Q

552  AAATTGATAA TGCTAAACTG GCTGCTGAGG ACTTCAGACT GAA||GTATGAG
      I  D  N  A  K  L  A  A  E  D  F  R  L  K  Y  E

602  ACTGAGAGAG GAATACGTCT AACAGTGGAA GCTGATCTCC AAGGCCTGAA
     T  E  R  G  I  R  L  T  V  E  A  D  L  Q  G  L  N

652  TAAGGTCTTT GATGACCTAA CCCTACATAA AACAGATTTG GAGATTCAAA
      K  V  F  D  D  L  T  L  H  K  T  D  L  E  I  Q  I

702  TTGAAGAACT GAATAAAGAC CTAGCTCTCC TCAAAAAGGA GCATCAGGAG||
      E  E  L  N  K  D  L  A  L  L  K  K  E  H  Q  E

752  GAAGTCGATG GCCTACACAA GCATCTGGGC AACACTGTCA ATGTGGAGGT
     E  V  D  G  L  H  K  H  L  G  N  T  V  N  V  E  V

802  TGATGCTGCT CCAGGCCTGA ACCTTGGCGT CATCATGAAT GAAATGAGGC
      D  A  A  P  G  L  N  L  G  V  I  M  N  E  M  R  Q

852  AGAAGTATGA AGTCATGGCC CAGAAGAACC TTCAAGAGGC CAAAGAACAG
      K  Y  E  V  M  A  Q  K  N  L  Q  E  A  K  E  Q
```

Figure 5 (sheet 1 of 2)

```
902   TTTGAGAGAC AG||ACTGCAGT TCTGCAGCAA CAGGTCACAG TGAATACTGA
      F  E  R  Q    T  A  V  L  Q  Q  Q  V  T  V  N  T  E

952   AGAATTAAAA GGAACTGAGG TTCAACTAAC GGAGCTGAGA CGCACCTCCC
        E  L  K  G  T  E  V  Q  L  T  E  L  R  R  T  S  Q

1002  AGAGCCTTGA GATAGAACTC CAGTCCCATC TCAGCATG||AA AGAGTCTTTG
        S  L  E  I  E  L  Q  S  H  L  S  M  K  E  S  L

1052  GAGCACACTC TAGAGGAGAC CAAGGCCCGT TACAGCAGCC AGTTAGCCAA
      E  H  T  L  E  E  T  K  A  R  Y  S  S  Q  L  A  N

1102  CCTCCAGTCG CTGTTGAGCT CTCTGGAGGC CCAACTGATG CAGATTCGGA
        L  Q  S  L  L  S  S  L  E  A  Q  L  M  Q  I  R  S

1152  GTAACATGGA ACGCCAGAAC AACGAATACC ATATCCTTCT TGACATAAAG
        N  M  E  R  Q  N  N  E  Y  H  I  L  L  D  I  K

1202  ACTCGACTTG AACAGGAAAT TGCTACTTAC CGCCGCCTTC TGGAAGGAGA
      T  R  L  E  Q  E  I  A  T  Y  R  R  L  L  E  G  E

1252  AGACGTAAA||A ACTACAGAAT ATCAGTTAAG CACCCTGGAA GAGAGAG||ATA
        D  V  K    T  T  E  Y  Q  L  S  T  L  E  E  R  D  I

1302  TAAAGAAAAC CAGGAAGATT AAGACAGTCG TGCAAGAAGT AGTGGATGGC
        K  K  T  R  K  I  K  T  V  V  Q  E  V  V  D  G

1352  AAGGTCGTGT CATCTGAAGT CAAAGAGGTG GAAGAAAATA TC|TAA||ATAGC
      K  V  V  S  S  E  V  K  E  V  E  E  N  I  *

1402  TACCAGAAGG AGATGCTGCT GAGGTTTTGA AAGAAATTTG GCTATAATCT

1452  TATCTTTGCT CCCTGCAAGA AATCAGCCAT AAGAAAGCAC TATTAATACT

1502  CTGCAGTGAT TAGAAGGGGT GGGGTGGCGG GAATCCTATT TATCAGACTC

1552  TGTAATTGAA TATAAATGTT TTACTCAGAG GAGCTGCAAA TTGCCTGCAA

1602  AAATGAAATC CAGTGAGCAC TAGAATATTT AAAACATCAT TACTGCCATC

1652  TTTATCATGA AGCACATCAA TTACAAGCTG TAGACCACCT AATATCAATT

1702  TGTAGGTAAT GTTCCTGAAA ATTGCAATAC A TTCAATTA TACTAAACCT

1752  CACAAAGTAG AGGAATCCAT GTAAATTGCA AATAAA
```

Figure 5 (sheet 2 of 2)

1 T

2 GTCAGCAGAG GAGGAGTTTC TTGCCTGTGG ACTTCATAAA AGGCTAGCTC

52 AACACCCTCC ATGAGACACA CTCTGCCCCA ACCATCCTGA AGCTACAGGT

102 GCTCCCTCCT GGAATCTCCA ATGGATTTCA GTCGCAGAAG CTTCCACAGA
M D F S R R S F H R

152 AGCCTGAGCT CCTCCTTGCA GGCCCCTGTA GTCAGTACAG TGGGCATGCA
S L S S S L Q A P V V S T V G M Q

202 GCGCCTCGGG ACGACACCCA GCGTTTATGG GGGTGCTGGA GGCCGGGGCA
R L G T T P S V Y G G A G G R G I

252 TCCGCATCTC CAACTCCAGA CACACGGTGA ACTATGGGAG CGATCTCACA
R I S N S R H T V N Y G S D L T

302 GGCGGCGGGG ACCTGTTTGT TGGCAATGAG AAAATGGCCA TGCAGAACCT
G G G D L F V G N E K M A M Q N L

352 AAATGACCGT CTAGCGAGCT ACCTAGAAAA GGTGCGGACC CTGGAGCAGT
N D R L A S Y L E K V R T L E Q S

402 CCAACTCCAA ACTTGAAGTG CAAATCAAGC AGTGGTACGA AACCAACCGC
N S K L E V Q I K Q W Y E T N R

452 CCGAGGGCTG GTCGCGACTA CAGTGCATAT TACAGACAAA TTGAAGAGCT
P R A G R D Y S A Y Y R Q I E E L

502 GCGAAGTCAG||ATTAAGGATG CTCAACTGCA AAATGCTCGG TGTGTCCTGC
R S Q I K D A Q L Q N A R C V L Q

552 AAATTGATAA TGCTAAACTG GCTGCTGAGG ACTTCAGACT GAA||GTATGAG
I D N A K L A A E D F R L K Y E

602 ACTGAGAGAG GAATACGTCT AACAGTGGAA GCTGATCTCC AAGGCCTGAA
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652 TAAGGTCTTT GATGACCTAA CCCTACATAA AACAGATTTG GAGATTCAAA
K V F D D L T L H K T D L E I Q I

702 TTGAAGAACT GAATAAAGAC CTAGCTCTCC TCAAAAAGGA GCATCAGGAG||
E E L N K D L A L L K K E H Q E

752 GAAGTCGATG GCCTACACAA GCATCTGGGC AACACTGTCA ATGTGGAGGT
E V D G L H K H L G N T V N V E V

802 TGATGCTGCT CCAGGCCTGA ACCTTGGCGT CATCATGAAT GAAATGAGGC
D A A P G L N L G V I M N E M R Q

Figure 6 (sheet 1 of 2)

852 AGAAGTATGA AGTCATGGCC CAGAAGAACC TTCAAGAGGC CAAAGAACAG
K Y E V M A Q K N L Q E A K E Q

902 TTTGAGAGAC AG||ACTGCAGT TCTGCAGCAA CAGGTCACAG TGAATACTGA
F E R Q T A V L Q Q Q V T V N T E

952 AGAATTAAAA GGAACTGAGG TTCAACTAAC GGAGCTGAGA CGCACCTCCC
E L K G T E V Q L T E L R R T S Q

1002 AGAGCCTTGA GATAGAACTC CAGTCCCATC TCAGCATG||AA AGAGTCTTTG
S L E I E L Q S H L S M K E S L

1052 GAGCACACTC TAGAGGAGAC CAAGGCCCGT TACAGCAGCC AGTTAGCCAA
E H T L E E T K A R Y S S Q L A N

1102 CCTCCAGTCG CTGTTGAGCT CTCTGGAGGC CCAACTGATG CAGATTCGGA
L Q S L L S S L E A Q L M Q I R S

1152 GTAACATGGA ACGCCAGAAC AACGAATACC ATATCCTTCT TGACATAAAG
N M E R Q N N E Y H I L L D I K

1202 ACTCGACTTG AACAGGAAAT TGCTACTTAC CGCCGCCTTC TGGAAGGAGA
T R L E Q E I A T Y R R L L E G E

1252 AGACGTAAA||A ACTACAGAAT ATCAGTTAAG CACCCTGGAA GAGAGAG||ATA
D V K T T E Y Q L S T L E E R D I

1302 TAAAGAAAAC CAGGAAGATT AAGACAGTCG TGCAAGAAGT AGTGGATGGC
K K T R K I K T V V Q E V V D G

1352 AAGGTCGTGT CATCTGAAGT CAAAGAGGTG GAAGAAAATA TC|TAA||ATAGC
K V V S S E V K E V E E N I *

1402 TACCAGAAGG AGATGCTGCT GAGGTTTTGA AAGAAATTTG GCTATAATCT

1452 TATCTTTGCT CCCTGCAAGA AATCAGCCAT AAGAAAGCAC TATTAATACT

1502 CTGCAGTGAT TAGAAGGGGT GGGGTGGCGG GAATCCTATT TATCAGACTC

1552 TGTAATTGAA TATAAATGTT TTACTCAGAG GAGCTGCAAA TTGCCTGCAA

1602 AAATGAAATC CAGTGAGCAC TAGAATATTT AAAACATCAT TACTGCCATC

1652 TTTATCATGA AGCACATCAA TTACAAGCTG TAGACCACCT AATATCAATT

1702 TGTAGGTAAT GTTCCTGAAA ATTGCAATAC A TTCAATTA TACTAAACCT

1752 CACAAAGTAG AGGAATCCAT GTAAATTGCA AATAAA

Figure 6 (sheet 2 of 2)

1	GAAAAGGTGC	GGACCCTGGA	GCAGTCCAAC	TCCAAACTTG	AAGTGCAAAT
51	CAAGCAGTGG	TACGAAACCA	ACGCCCCGAG	GGCTGGTCGC	GACTACAGTG
101	CATATTACAG	ACAAATTGAA	GAGCTGCGAA	GTCAGATTAA	GGATGCTCAA
151	CTGCAAAATG	CTCGGTGTGT	CCTGCAAATT	GATAATGCTA	AACTGGCTGC
201	TGAGGACTTC	AGACTGAAGT	ATGAGAGTGA	GAGAGGAATA	CGTCTAACAG
251	TGGAAGCTGA	TCTCCAAGGC	CTGAATAAGG	TCTTTGATGA	CCTAACCCTA
301	CATAAAACAG	ATTTGGAGAT	TCAAATTGAA	GAACTGAATA	AAGACCTAGC
351	TCTCCTCAAA	AAGGAGCATC	AGGAGGAAGT	CGATGGCCTA	CACAAGCATC
401	TGGGCAACAC	TGTCAATGTG	GAGGTTGATG	CTGCTCCAGG	CCTGAACCTT
451	GGCGTCATCA	TGAATGAAAT	GAGGCAGAAG	TATGAAGTCA	TGGCCCAGAA
501	GAACCTTCAA	GAGGCCAAAG	AACAGTTTGA	GAGACAGACT	GCAGTTCTGC
551	AGCAACAGGT	CACAGTGAAT	ACTGAAGAAT	TAAAAGGAAC	TGAGGTTCAA
601	CTAACGGAGC	TGAGACGCAC	CTCCCAGAGC	CTTGAGATAG	AACTCCAGTC
651	CCATCTCAGC	ATGAAAGAGT	CTTTGGAGCA	CACTCTAGAG	GAGACCAAGG
701	CCCGTTACAG	CAGCCAGTTA	GCCAACCTCC	AGTCGCTGTT	GAGCTCTCTG
751	GAGGCCCAAC	TGATGCAGAT	TCGGAGTAAC	ATGGAACGCC	CGAACAACGA
801	ATACCATATC	CTTCTTGACA	TAAAGACTCG	ACTTGAACAG	GAAATTGCTA
851	CTTACCGCCG	CCTTCTGGAA	GGAGAAGACG	TAAAAACTAC	AGAATATCAG
901	TTAAGCACCC	TGGAAGAGAG	AGATATAAAG	AAAACCACCA	AGATTAAGAC
951	AGTCGTGCAA	GAAGTAGTGG	ATGGCAAGGT	CGTGTCATCT	GAAGTCAAAG
1001	AGGTGGAAGA	AAATATCTAA	ATAGCTACAG	AAGGAGATGC	TGCTGAGGTT
1051	TTGAAAGAAA	TTTGGCTATA	ATCTTATCTT	TGCTCCCTGC	AAGAAATCAG
1101	CCATAAGAAA	GCACTATTAA	TACTCTGCAG	TGATTAGAAG	GGGTGGGGTG
1151	GCGGGAATCC	TATTTATCAG	ACTCTGTAAT	TGAATATAAA	TGTTTTACTC
1201	AGAGGAGCTG	CAAATTGCCT	GCAAAAATGA	AATCCAATGA	GCACTAGAAT
1251	ATTTAAAACA	TCATTACTGC	CATCTTTATC	ATGAAGCACA	TCAATTACAA
1301	GCTGTAGACC	ACCTAATATC	AATTTGTAGG	TAATGTTCCT	GAAAATTGCA
1351	ATACATTTCA	ATTATACTAA	ACCTCACAAA	GTAGAGGAAT	CCATGTAAAT
1401	TGCAAATAAA	CCACTTTCTA	ATTTTTAAAA	AAAAAAAAAA	AAAAAAAAAA
1451	AAAAAAAAAA	A			

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Figure 7

International Application No
PCT/[illegible] 95/02734

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12P19/34 C07H21/04 G01N33/68 //C07K14/47, C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF CANCER, vol. 71, no. 2, February 1995 pages 278-81, BURCHILL, S. ET AL. 'detection of epithelial cancer cells in peripheral blood by reverse transcriptase-polymerase chain reaction ' see the whole document ---	1-12
X	WO,A,92 02558 (PROGEN BIOTECHNIK GMBH) 20 February 1992 see claim 27; example 12 ---	1-5

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 February 1996	19.03.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y		6-12
Y	JOURNAL OF CLINICAL ONCOLOGY, vol. 12, no. 3, March 1994 pages 475-82, DATTA, Y. ET AL 'Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction' see the whole document	6-12
Y	CANCER RESEARCH, vol. 54, June 1994 pages 2986-90, SCHOENFIELD, A. ET AL 'detection of breast cancer micrometastases in auxillary nodes by using polymerase chain reaction' see the whole document	6-12

2

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		EP-A-	0541645	19-05-93
		JP-T-	6501924	03-03-94